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Rapid detection and identification of *Streptococcus iniae* using a monoclonal antibody-based indirect fluorescent antibody technique

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Abstract

Streptococcus iniae is among the major pathogens of a large number of fish species cultured in fresh and marine recirculating and net pen production systems. The traditional plate culture technique to detect and identify *S. iniae* is time consuming and may be problematic due to phenotypic variations of *S. iniae* isolates. The rapid transmission and severe economic impact of this disease necessitates the development of a rapid, reliable, specific, and sensitive technique to detect and identify *S. iniae*. We developed an indirect fluorescent antibody technique (IFAT) based on a highly specific mononclonal antibody for *S. iniae*. The IFAT was found to be suitable for the detection and identification of *S. iniae* from experimentally and naturally infected tilapia (*Oreochromis niloticus*). The olfactory epithelium of the naris of experimentally and naturally infected tilapia was demonstrated to be a reliable, sensitive, and non-lethal sample site for the detection and identification of *S. iniae*. This IFAT was validated by the traditional plate culture techniques but found to be considerably less time consuming and problematic. These findings reveal that this IFAT is an improvement in the ability to detect and identify *S. iniae* in infected and carrier fish.

Keywords: *Streptococcus iniae*; Diagnosis; Indirect fluorescent antibody technique; Monoclonal antibody; Infection; Carrier; Naris

1. Introduction

Streptococcus iniae is a Gram-positive bacterium that causes significant disease problems in fresh and marine water fish. Streptococcal disease is one of the most common bacterial diseases in hybrid striped bass

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(*Morone chrysops* × *Morone saxatilis*) (Stoffregen et al., 1996; Shoemaker et al., 2001); tilapia (*Oreochromis niloticus*) (Kitao et al., 1981; Shoemaker and Klesius, 1997; Bowser et al., 1998; Shoemaker et al., 2001); (*O. niloticus* × *Oreochromis aurea*) (Perera et al., 1994, 1997); rainbow trout (*Oncorhynchus mykiss*) (Kitao et al., 1981; Eldar et al., 1995); red drum (*Sciaenops ocellatus*) (Eldar et al., 1999); yellowtail (*Seriola quinquerodiata*) (Kaige et al., 1984); rabbitfish (*Siganus canaliculatus*) (Yuasa et al., 1999); sea bass (*Dicentrarchus labrax*) (Colorni et al., 2002); Japanese flounder (*Paralichthys olivaceus*) (Nguyen et al., 2002); ayu (*Plecoglossus altivelis*) (Kitao et al., 1981); and barramundi (*Lateus calcifer*) (Bromage et al., 1999). This disease causes significant economic losses in the aquaculture industry in the United States of America, Japan, Israel, South Africa, Australia, the Philippines, Taiwan, Bahrain, and other countries.

Current detection and identification techniques for *Streptococcus* sp. are based on plate cultivation, biochemical techniques, enzyme reactions, Biolog[1] phenotypic analysis, and whole cell fatty acid analysis (Facklam and Washington, 1991; Shoemaker and Klesius, 1997; Shoemaker et al., 2000; Evans et al., 2002). However, these techniques are time consuming and phenotypic variation of *S. iniae* isolates may make timely identification with biochemical and enzymatic techniques problematic. The identification of *S. iniae* using API 20 Strep and rapid ID 32 Strep systems (BioMérieux, Hazelwood, MO) is not acceptable due to lack of proper phenotypic tests and the absence of *S. iniae* catalogued in their databases. Furthermore, the isolation and identification of *S. iniae* may also be problematic, especially from the aquaculture environment (Nguyen et al., 2002). Enrichment techniques have improved the detection of *S. iniae* (Shoemaker et al., 2001; Nguyen et al., 2002) but not the length of time required to correctly identify *S. iniae*. In addition, carriers may be extremely difficult to detect due to low numbers of *S. iniae* cells and unknown tissue location of *S. iniae* in the carrier.

These facts led us to the possibility that IFAT based on a specific *S. iniae* monoclonal antibody (MAb) might be able to rapidly detect *S. iniae* from the nares of infected and carrier fish. The objective of this study was to develop a rapid and non-lethal technique to detect and identify *S. iniae*, thereby providing an improved diagnostic technique for this major fish pathogen.

2. Materials and methods

2.1. S. iniae isolates and isolates other than S. iniae

In order to investigate the *in vitro* validation and determine the specificity and sensitivity of the IFAT, a wide variety of *S. iniae* from different sources were used (Table 1). The *S. iniae* isolates and other bacterial species were obtained from recognized culture collections. Reference cultures, isolates from clinical fish cases, and isolates from non-fish sources were tested. These bacteria were grown on 5% sheep blood agar (Remel Lexena, KS) following incubation aerobically for 20–24 h at 27 °C.

2.2. Plate culture and identification techniques

The plate culture techniques (PCT) employed to validate the IFAT were those previously employed for *S. iniae* (Shoemaker et al., 2001). Briefly, swab samples were taken aseptically from the left eye (retro-orbitally), naris olfactory epithelium, brain cerebellum, and anterior kidney of experimental and naturally infected tilapia for plate culture technique. The swabs were dropped into 5 mL of tryptic soy broth (TSB, Difco, Detroit, MI), supplemented with defibrinated sheep blood (5 drops) and incubated for 3–4 h at 27 °C. Broth samples were then streaked on tryptic soy agar plates (TSA, Remel) and incubated aerobically for 20–24 h at 28 °C. Pure cultures were then sampled and subjected to morphologic and cellular fatty acid analysis.

Cellular morphology and Gram stain were determined for pure colonies that were characteristic of *S. iniae*. One isolate per plate was subjected to quantitative analysis of cellular fatty acid compositions performed using the gas–liquid chromatographic (GC) procedure as described by the Microbial Identification System (MIS, MIDI Inc., Newark, DE). The cultures were grown on TSA for 24 h at 28 °C. The

[1] Use of trade or manufacturer name does not imply endorsement by the USDA Department of Agriculture.

Table1
Bacterial species, isolates, origin, culture designation and the indirect fluorescent antibody technique (IFAT) results

Bacterial species and isolates	Origin	Culture collection and isolate number	IFAT results[a]
Streptococcus iniae	Hybrid striped bass *Morone chrysops* × *Morone saxatilis*	AAHRL[b] ARS-56	Positive
	Hybrid striped bass	AAHRL ARS-57	Positive
	Hybrid striped bass	AAHRL ARS-60	Positive
	Hybrid striped bass	AAHRL ARS-JC	Positive
	Tilapia *Oreochromis niloticus*	AAHRL ARS-CIB	Positive
	Tilapia	AAHRL ARS-T23D25	Positive
	Tilapia	AAHRL ARS-M6	Positive
	Red tail black shark *Epalzeorhynchos biocolor*	AAHRL ARS-PB-03-62	Positive
	Amazon river dolphin *Inia geoffrensis*	ATCC[c] 29177	Positive
	Amazon river dolphin	ATCC 29178	Positive
	Human *Homo sapiens*	CDC[d] 4440	Positive
Streptococcus agalactiae	Mullet *Liza Klunzingeri*	AAHRL ARS-MU2N	Negative
	Mullet	AAHRL ARS-MU11B	Negative
	Gilthead seabream *Sparus auratus* L.	AAHRL ARS-37B	Negative
Streptococcus epidermis	Channel catfish *Ictalurus punctatus*	AAHRL ARS-265	Negative
Streptococcus pyogenes	Human	CECT[e] 985	Negative
Streptococcus phocae	Harbor seal *Phoca vitulina*	ATCC 51973	Negative
Streptococcus dysgalactiae	Bovine *Bos taurus*	ATCC 13813	Negative
Streptococcus uberis	Pig *Suis scrofa domesticus*	ATCC 700407	Negative
Lactococcus lactis	Cheese	CECT 540	Negative
Lactococcus garvieae	Yellowtail *Seriola quinqueradiata*	CECT 4828	Negative
	Bovine	CECT 4531	Negative
Staphylococcus aureus	Unknown	CECT 59	Negative
Micrococcus luteus	Soil	CECT 241	Negative
Enterococcus faecalis	Bovine milk	CECT 184	Negative
Aeromonas hydrophila	Channel catfish	AAHRL ARS-AER003	Negative

[a] IFAT of equal or greater than 10–20 fluorescent *S. iniae* cells/field.
[b] AAHRL designates the Aquatic Animal Health Research Laboratory Culture Collection.
[c] ATCC designates the American Type Culture Collection.
[d] CDC designates Centers for Disease Control and Prevention Culture Collection.
[e] CECT designates the Colecciõn Española de Cultivos Tipo (Spanish Type Culture Collection).

colonies were harvested and 20 ± 0.5 mg of cells were placed in glass screw cap test tubes. The sample tubes received 1 mL of strong methanolic base (45 g NaOH, 250 mL methanol, and 150 mL distilled water). The tubes were vortexed and then heated for 5 min in a 100 °C water bath then revortexed and again heated for 10 min in a 80 °C water bath. The cooled sample mixtures were extracted for fatty acid methyl esters with 1.25 mL of extraction mixture (200 mL hexane, 200 mL methyl *tert*-butyl ether) and mixed for 10 min. The top phase was removed and 3 mL of a base wash was added (10.8 g sodium hydroxide and 900 mL distilled water). The mixtures were again mixed as above for 5 min and the top phase was removed and placed into GC sample vials. The methyl fatty acid esters were analyzed with an Agilent Technologies (Palo Alto, CA) model 6850 GC equipped with a 7683 autoinjector, split–splitless inlet with a Merlin microseal septum (Merlin Instruments Company, Half Moon Bay, CA), flame ionization detector, and a 25-m-long × 0.33-μm film thickness Ultra 2 column. The isolate samples were identified for genus and species using the Sherlock RCLIN50 method (MIS).

2.3. Indirect fluorescence antibody (IFAT) technique

Fifteen-millimeter circles on pre-cleaned Fluoro slides (Eric Scientific, Portsmouth, NH) to be processed for IFAT were smeared with sterile swabs or

inoculating loops taken from the target tissues and pure plate cultures. The smears were air-dried prior to heat fixation by several passages of the slides over an open flame. The fixed smears were treated with 25 μL of 1:20 dilution of MAb (P11 Batch #0306/P11/01, anti-*S. iniae*, Aquatic Diagnostics Ltd, Institute of Aquaculture, University of Stirling, Stirling, Scotland,) in phosphate-buffered saline (PBS) for 1 h at room temperature. The slides were washed three times in 0.1% Tween-20 PBS, drained between washings, and incubated with 25 μL of 1:4000 dilution of monoclonal anti-mouse fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich, St. Louis, MO) in PBS for 1 h in the dark at room temperature. Slides were washed as above, drained, air-dried, and cover slipped (No. 1 22 × 50-nm cover slip, Corning Inc., Acton, MA) using one drop of Cytoseal mounting medium (Richard Allan Scientific, Kalamazoo, MI) as a mountant. The cover slips were allowed to harden by sitting for 1 h in a dark container at room temperature. Fluorescent antibody-reactive bacteria were scored using a Zeiss fluorescent microscope (Zeiss, Thornwood, NY) with ×40 objective. The positive samples had equal or greater than 10–20 fluorescent bacterial cells/field and negative samples had no detectable fluorescent bacterial cells/field.

2.4. S. iniae experimental infection of tilapia

The inoculum was prepared by incubating 250 mL broth cultures of frozen *S. iniae* ARS-60 isolate in 500-mL culture flasks. Twenty-five Nile tilapia (15–20 g) were intraperitoneally (i.p.) injected with 0.1 mL of the *S. iniae* culture at 3.5×10^7 CFU/fish. An additional 25 Nile tilapia (15–20 g) were used as non-infected control fish and were i.p. injected with 0.1 mL of TSB. The two groups of fish were maintained separately in 57-L glass aquaria. The aquaria were supplied with 0.5 L/h flow-through water and aerated with an air stone. The fish were fed daily to satiation with Aquamax Grower (Brentwood, MO). A light/dark period of 12:12 h was maintained. Mortality was monitored daily for 10 days post-challenge and dead fish were removed daily; paired swab samples were taken from the eye, naris, brain, and kidney for IFAT and PCT analysis, respectively, as described above. No infected fish died after 8 days post-challenge. Signs of disease that were observed in the infected fish included erratic swimming, whirling, eye opacity, body curvature, refusal of food, and darkening of skin pigmentation. No mortality or signs of disease were observed in the non-infected fish.

A third group of 25 Nile tilapia (15–20 g) were inoculated i.p. with *S. iniae* and paired nare swab samples were taken for IFAT and PCT analysis as described above at 1 day post-challenge. No signs of disease or mortality were observed in these fish.

2.5. S. iniae naturally infected tilapia

Thirty-three moribund tilapia (225 g) were obtained from a raceway on a private farm in Arkansas that had been experiencing daily mortalities of approximately 100 fish caused by *S. iniae* infection. The fish were grown in a 39,630-L outdoor in-ground raceway in a recirculating system without adequate biofiltration and gas stripping. Many fish exhibited signs of disease that included erratic swimming, whirling, eye opacity, body curvature, refusal of food, and darkening of skin pigmentation. The fish were collected, frozen, and shipped to the USDA, ARS Aquatic Animal Health Research Laboratory, Auburn, AL, for the purpose of detection and identification of *S. iniae* in their tissues by IFAT and PCT.

3. Results

3.1. Cellular fatty acid analysis for S. iniae

Fatty acid analysis showed that in the 11 culture collection isolates (Table 1), streptococcal isolates from the experimental and the naturally infected tilapia were *S. iniae*. Seven major different fatty acids (>90% of the fatty acid profiles) were found, with only three being saturated. The principal fatty acids were 14:0, 15:0 iso2 OH/16:1w7c, 16:0, 18:1 w9c, 18:1 w7c, 18:0, and 19:0 cyclo w8c. The similarity indexes were 0.5 or greater as compared to standard library cultures.

3.2. IFAT specificity and sensitivity

In order to determine the specificity and sensitivity of the IFAT, isolates of *S. iniae* and other bacterial

species from a variety of sources and culture collections, including the reference strains, were tested (Table 1). The IFAT correctly identified the 11 isolates of *S. iniae* that had been previously identified as *S. iniae*. The IFAT did not yield any false positive results with related or non-related bacterial species that include *Streptococcus agalactiae*, *Streptococcus epidermis*, *Streptococcus pyogenes*, *Streptococcus phocae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Lactococcus lactis*, *Lactococcus garvieae*, *Staphylococcus aureus*, *Micrococcus luteus*, *Enterococcus faecalis*, and *Aeromonas hydrophila*.

3.3. Validation of the IFAT by the traditional PCT

The IFAT and PCT successfully detected and identified *S. iniae* in the eye, nare, brain, and kidney samples obtained from four dead experimentally infected fish between 2 and 6 days post-challenge (Table 2). The only exception was that the IFAT did not detect and identify *S. iniae* in the eye of one fish, whereas the PCT did at 3 days post-challenge. The nare samples yielded positive results by both IFAT and PCT at 7 days post-challenge. The numbers of positive samples from the eye, brain, and kidney declined at 7 days post-challenge. The presence of *S. iniae* in nare samples from three out of four dead fish was detected by IFAT at 8 days post-challenge. The PCT identified two out of these four fish as positive from eye samples at 8 days post-challenge. The presence of *S. iniae* in brain samples was more readily identified by the IFAT than by the PCT at 8 days post-challenge. The kidney samples taken at 8 days post-challenge from four dead fish were IFAT and PCT positive. The overall results indicated that IFAT and PCT were in close agreement using nare and brain samples from the 13 of 25 fish that died during the challenge. The presence of *S. iniae* was not identified in any of the tissue samples from 25 non-challenged control fish by either IFAT or PCT.

The IFAT was 100% successful in the detection and identification of *S. iniae* in the eye, nare, and brain samples from 33 thawed tilapia that had been freshly collected and frozen during a natural outbreak of *S. iniae* (Table 2). The results of the IFAT and PCT were 100% in agreement. However, no agreement was noted between IFAT and PCT using kidney samples. Correct detection and identification of *S. iniae* was 33% and 82% by IFAT and PCT, respectively, from these kidney samples.

In order to determine the ability of the IFAT to correctly detect and identify *S. iniae* in asymptomatic tilapia, the naris of 25 infected tilapia were non-lethally sampled at 1 day post-challenge. A total of 10 out of 25 nare samples were identified as being *S. iniae* positive by IFAT and PCT. However, the PCT failed to identify one fish positive by IFAT, and the IFAT failed to identify one fish positive by PCT. The number of positive and negative samples was 9 and 14, respectively, for both IFAT and PCT.

Table 2
Percent positive tissues for *Streptococcus iniae* by indirect fluorescent antibody technique (IFAT) and plate culture technique (PCT) from *S. iniae* experimentally, naturally infected, and non-infected control tilapia

Infection	Percent positive tissues by IFAT/PCT[a]					
	Days post-challenge	Number of morbid fish	Eye	Naris	Brain	Kidney
Experimental[b]	2	1	100/100	100/100	100/100	100/100
	3	2	50/100	100/100	100/100	100/100
	6	1	100/100	100/100	100/100	100/100
	7	5	60/60	100/100	80/100	40/60
	8	4	75/50	75/50	100/75	100/100
	All days	13	77/82	95/90	96/95	88/92
Non-infected controls[c]	8	0	0/0	0/0	0/0	0/0
Natural[d]	NA	33	100/100	100/100	100/100	33/82

[a] IFAT of equal or greater than 10–20 fluorescent *S. iniae* cells/field and PCT for *S. iniae* colonies identified by whole cell fatty acid analysis.
[b] Morbid tilapia from 25 tilapia experimentally infected with *S. iniae* 3.5×10^7 CFU/fish by i.p. injection.
[c] Tissue samples from 25 non-infected tilapia at 8 days.
[d] Tissue samples from 33 frozen and thawed tilapia collected from a recirculating tilapia culture facility experiencing a natural outbreak of *S. iniae*.

4. Discussion

In this study, the results demonstrated the validity, specificity, and sensitivity of IFAT to detect and identify *S. iniae* in various tissues of experimentally and naturally infected tilapia. The results of the IFAT and the more traditional PCT for the detection and identification of *S. iniae* were in agreement for both experimentally and naturally infected fish. Previously, an IFAT was described for the identification of streptococcosis in rainbow trout (Bragg, 1988). A rabbit antiserum was produced against a single streptococcal species isolate. This primary antiserum was used to recognize streptococcosis caused by biochemically identical isolates in 25 diseased trout and experimentally infected trout. The IFAT was reported to be positive for spleen, liver, and kidney samples from infected fish. Pure cultures of *Enterococus faecium*, *L. lactis*, and *A. hydrophila* were IFAT negative. The identification of the species of the *Streptococcus* isolate was not reported. Furthermore, no evidence of validation using the traditional PCT was presented for this rabbit antibody IFAT. In contrast, we used a MAb that is highly specific for *S. iniae* in our IFAT. The specificity and sensitivity of this MAb was demonstrated using pure cultures obtained from a bacterial culture repository that included the reference isolates for *S. iniae*.

Recently, Nguyen et al. (2002) suggested the permanent presence of *S. iniae* on a farm culturing Japanese flounder. Water and sediment samples were found to be culture positive for *S. iniae*. The culture water was pumped and discharged from the same seawater body. The isolation rate for the water supply and sediment was highest in warmer months which closely coincided with the outbreaks of clinical disease. Contamination of the sea water and sediment may be a result of *S. iniae* being able to replicate in the intestinal tract of fish and be released into water through fecal waste (Bromage et al., 1999; McNulty et al., 2003).

Immersion of fish in water containing *S. iniae* (Perera et al., 1997; Bromage et al., 1999) and cohabitation with diseased fish (Shoemaker et al., 2000) have resulted in experimental infections. Recently, Bromage and Owens (2002) proposed *S. iniae* infection occurred orally through the consumption of infected carrier fish or through the faecal–oral route. Furthermore, water-borne infection via nares (Evans et al., 2000, 2001) gills (McNulty et al., 2003) and through damaged skin, especially at higher fish densities and challenge doses (Shoemaker et al., 2000), are among the most likely routes of transmission. We also have observed the permanent presence of *S. iniae* and carriers in recirculating fish production systems in the U.S. (Shoemaker et al., 2001, Evans et al., preliminary data, KentSea Tech. Report).

The naturally infected tilapia reported here were from a farm that produced the fish in an outdoor raceway at a high density (100,000 fish at 225 g in a water volume of 39,630 L). The water supply was recirculated without an adequate biofilter system. The average daily mortality was 100 fish and the *S. iniae* outbreak was initiated at the beginning of the warm season. The ecological factors, production conditions, and epidemiology of *S. iniae* at this tilapia farm are similar to those reported at a Japanese flounder farm having outbreaks of *S. iniae* (Nguyen et al., 2002).

Previously, we had demonstrated that the naris was readily infected with *S. iniae* (Evans et al., 2000, 2001). We hypothesized that the nares may provide a reliable and non-lethal sample site for detection and identification of *S. iniae* in naturally and experimentally infected fish (Evans et al., 2001). Furthermore, we also previously demonstrated that streptococci can survive in the tissues of frozen fish following natural and experimental infection including the nares tissue (Evans et al., 2004). The results of this study proved that *S. iniae* may be rapidly and reliably detected and identified in nares samples from fresh and frozen infected fish within 24 h. The IFAT described in this study may be very useful for the detection and identification of *S. iniae* in asymptomatic carriers and infected fish.

The IFAT is a sensitive technique with the potential, after further study, to identify *S. iniae* from culture water, sediment, culture equipment, fish feed, and the skin of fish. The IFAT can to detect and identify as few as 10 *S. iniae* cells. Fewer than 50 *S. iniae* cells are less likely to be recovered using the traditional PCT. The highly infectious nature and severe economic impact of *S. iniae* disease warrants a rapid and reliable detection and identification technique that is fundamental to effective management decisions on how to control this disease in recirculating water and cage production systems, especially

during the warmer seasons in the temperate regions and in the tropical regions of the world. In conclusion, this IFAT used to rapidly detect and identify *S. iniae* may improve management decisions and thus reduce the negative economic impact of this disease in aquaculture production systems.

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